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Expression of Calcineurin B Homologous Protein 2 Protects Serum Deprivation-induced Cell Death by Serum-independent Activation of Na^+/H^+ Exchanger*

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The calcineurin B homologous protein (designated CHP1) has been shown to be a common essential cofactor for the plasma membrane Na^+/H^+ exchangers (NHEs) (Pang, T., Su, X., Wakabayashi, S., and Shigekawa, M. (2001) *J. Biol. Chem.* 276, 17367–17372). In this study, we characterized the function of another isoform of CHP (designated CHP2) that has a 61% amino acid identity with CHP1. CHP2, like CHP1, conferred the ability to NHEs 1–3 to express a high exchange activity by binding to the juxtamembrane region of the cytoplasmic domain of the exchanger, but it interacts more strongly (~5-fold) with NHE1 than does CHP1. Although CHP1 is expressed ubiquitously at relatively high levels, CHP2 expression was extremely low in most human tissues but was higher in tumor cells. We produced stable cell clones overexpressing either CHP1 or CHP2 in which one of them is predominantly bound to NHE1. Serum (10%) induced a significant cytoplasmic alkalinization (0.1–0.2 pH unit) in cells co-expressing CHP1 and NHE1 but not in cells co-expressing CHP2 and NHE1. In the latter, pH_i was high (7.4–7.5) even in the absence of serum, suggesting that NHE1 was already activated. Surprisingly, most (>80%) of CHP2/NHE1 cells unlike CHP1/NHE1 cells were viable even after long serum starvation (>7 days). Thus, the expression of CHP2 appears to protect cells from serum deprivation-induced death by increasing pH_i. These properties of CHP2/NHE1 cells are similar to those of malignantly transformed cells. We propose that serum-independent activation of NHE1 by bound CHP2 is one of the key mechanisms for the maintenance of high pH_i and the resistance to serum deprivation-induced cell death in malignantly transformed cells.

The Na^+/H^+ exchanger (NHE)[1] is an electroneutral countertransporter that catalyzes H^+ extrusion coupled to Na^+ influx across the biological membranes. The NHE family consists of at least seven isoforms that are different in tissue or subcellular localization (1–3). The ubiquitous NHE1 isoform plays a major role in intracellular pH (pH_i) homeostasis and cell volume regulation (1–3) and has extensively been studied in terms of its structure, function, and regulatory mechanism. An outstanding feature of NHE1 is that it is activated in response to various extracellular stimuli including hormones, growth factors, cytokines, and mechanical stress such as cell shrinkage, resulting in cytoplasmic alkalinization in the absence of bicarbonate (1–4). In the NHE1 regulation by these stimuli, the involvement of a variety of signaling molecules (*i.e.* calcineurin B homologous protein (5, 6), Ca^{2+}/calmodulin (7–9), low molecular weight GTPases Ras and Rho (10–12), p42/44 mitogen-activated protein kinases (13), p90 ribosomal S6 kinase (14), 14-3-3 protein (15), Nck-interacting kinase (16), and phosphatidylinositol 4,5-bisphosphate (17) has been reported. However, the interrelations among the functions of these signaling molecules leading to the NHE1 activation have not yet been well sorted out.

It has often been documented that the increased pH_i caused by NHE1 activation serves as a permissive or an obligatory signal for cell proliferation and differentiation (1–4, 18, 19). Oppositely, the decreased pH_i attributed to reduced NHE1 activity has been shown to result in growth arrest or cell death (20–23). Furthermore, the activation of NHE1 has often been associated with oncogenic transformation (24–28). For example, cells transformed by *ras* oncogene (25–27) or E7 oncogene from papillomavirus type 16 (28) have been shown to maintain high pH_i in the absence of serum with an accompanying high activity of NHE1, which may be one of key factors involved in abnormal cell growth or enhanced cell invasion. However, molecular mechanisms underlying these phenotypic alterations remain poorly understood.

Recently, we have provided evidence that calcineurin B homologous protein (CHP1) serves as an essential cofactor, which is required for at least three NHE isoforms, NHEs 1–3, to express high physiological levels of exchange activity because CHP1 deprivation results in dramatic reductions (>90%) of activity (6). However, it is not clear how CHP1 is involved in the regulation of NHEs in response to the extracellular stimuli. There is another human CHP isoform (CHP2) that was identified in human cancer patient (NCBI nucleotide accession number NM_022097 with designation of hepatocellular carcinoma antigen gene 520). CHP2 protein shares high homology with

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The amino acid sequence of this protein can be accessed through NCBI Protein Database under NCBI accession number NM_022097.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF146019.

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[1] The abbreviations used are: NHE, Na^+/H^+ exchanger; CHP, calcineurin B homologous protein; GFP, green fluorescent protein; MBP, maltose-binding protein; pH_i, intracellular pH; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; RT, reverse transcription.

CHP1 (61% amino acid identity). This prompted us to study functional differences between the two CHP isoforms, because such investigation would provide an important clue as to the role of CHP in the NHE1 regulation.

In this study, we found that cells co-expressing NHE1 and CHP2 but not cells co-expressing NHE1 and CHP1 maintain high pH_i through the activation of NHE1 even in the absence of serum. In addition, such cells remain viable even after serum starvation over 1 week. We conclude that the interaction of NHE1 with CHP2 but not with CHP1 leads to serum-independent permanent activation of NHE1, which is a well documented property found in malignantly transformed cells.

EXPERIMENTAL PROCEDURES

Antibodies and Other Materials—Polyclonal antibodies against NHE1 (RP-cd) and CHP1 (designated anti-CHP) were described previously (6). Anti-CHP antibody, which was produced by immunizing rabbit with glutathione *S*-transferase fusion protein containing a full-length CHP1, recognized both CHP1 and CHP2. To produce isoform-specific CHP antibodies (anti-CHP1 and anti-CHP2), we immunized rabbits with synthetic peptides (^{96}NEKSKDVNGP^{105} for human CHP1 and ^{96}EDTETQDPKKP^{106}for human CHP2, see Fig. 1*A*) conjugated with keyhole limpet hemocyanin. Immunoblot analysis using recombinant CHP1 or CHP2 proteins revealed that anti-CHP2 exclusively recognized CHP2, whereas anti-CHP1 recognized CHP1 and to a lesser extent CHP2. Various human malignantly transformed cell lines, *i.e.* hepatoma, colon adenocarcinoma, cervical carcinoma, and lymphocytic leukemia cells, were obtained from the Japan Health Sciences Foundation. Human fibroblasts were obtained from a skin biopsy sample. The amiloride derivative EIPA was a gift from New Drug Research Laboratories of Kanebo, Ltd. (Osaka, Japan). ^{22}NaCl and ^{14}C-benzoic acid were purchased from PerkinElmer Life Sciences. All other chemicals were of the highest purity available.

Cells Culture and Stable Expression—The Na^+/H^+ exchanger-deficient cell line (PS120) (29), the corresponding transfectants, human skin fibroblasts, and most of cancer cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 25 mM $NaHCO_3$ and supplemented with 7.5–10% (v/v) fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Leukemia cells were maintained in RPMI 1640 medium containing 10% serum. Cells were maintained at 37 °C in presence of 5% CO_2. PS120 cells (5×10^5 cells/100-mm dish) were transfected with each plasmid construct (20 μg) by the calcium phosphate co-precipitation technique. Cell populations stably expressing mutant NHE1 were selected by "H^+-killing" procedure as described previously (30). Cells stably overexpressing CHP1 or CHP2 were first selected with G418, and then single colonies were isolated by checking protein expression with immunoblot while cells expressing GFP-tagged CHP were selected with the aid of GFP fluorescence.

Construction of Expression Vectors—cDNAs of CHP1 and CHP2 were isolated by means of RT-PCR using cDNAs prepared from human blood or commercially available cDNAs (human MTC™ panel I, Clontech) as a template. A cDNA for NHE6 was kindly provided by Drs. M. Sakaguchi and K. Mihara (Kyushu University, Fukuoka, Japan). All the constructs were produced by means of the PCR-based strategy. GFP-untagged and tagged CHPs were constructed by inserting PCR fragments with and without a stop codon (TAA) into pEGFP-N1 (Clontech), respectively. Plasmids carrying cDNAs for human NHE1, rat NHE2, or NHE3 and their variants were all cloned into mammalian expression vector pECE. For construction of oocyte expression vectors, cDNAs for CHPs and NHEs were inserted into the modified pBluescript II containing poly(T^+). The cRNAs were synthesized with the mCAP™ RNA capping kit (Stratagene) using linearized DNA templates. Inserted DNA fragments were confirmed by sequencing plasmids with a DNA sequencer model 3100 (ABI) to ensure the fidelity of construction.

Purification of Recombinant Proteins and Pull-down Assay—For the production of recombinant CHP2 proteins, the DNA fragment was designed to contain six His residues and cloned into a bacterial expression vector pET11a (Stratagene), which was then expressed in *Escherichia coli* (BL21). For the production of MBP fusion proteins for human NHE1 or NHE6, the DNA fragments (amino acids 503–815 of NHE1 or amino acids 500–669 of NHE6) were incorporated into pMAL-c (New England Biolabs), and plasmids were incorporated into *E. coli* (HB101). Proteins were subsequently purified by using ProBond™ (Invitrogen) or amylose (New England Biolabs) resin column according to the manufacturer's protocol. For pull-down assay, CHP2 protein was incubated for 30 min at 4 °C with 30 μl of amylose resin pretreated with MBP-NHE fusion protein in Tris-buffered saline (150 mM NaCl and 10 mM Tris-Cl, pH 7.4). After washing five times, the protein was eluted from amylose resin with 50 mM maltose, electrophoresed, and then visualized by Coomassie Brilliant Blue staining.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were performed essentially as described previously (31). Cells were solubilized with 1% Triton X-100 containing 150 mM NaCl, 10 mM Hepes-Tris, pH 7.4, and protease inhibitors, and cell lysate was incubated with respective antibodies and protein A-Sepharose. After centrifugation, precipitated materials were separated on 7.5 or 12% polyacrylamide gels and transferred to Immobilon membranes (Millipore). After blocking, incubation with antibodies, and washing, protein signals were visualized with enhanced chemiluminescence (Amersham Biosciences).

Measurement of ^{22}Na Uptake—$^{22}Na^+$ uptake activity was measured by the potassium$^+$/nigericin pH_i clamp method (32). Serum-supplemented or depleted cells in 24-well dishes were preincubated for 30 min at 37 °C in Na^+-free choline chloride/potassium chloride medium containing 20 mM Hepes-Tris, pH 7.4, 1.2–140 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 5 μM nigericin. $^{22}Na^+$ uptake was started by adding the same choline chloride/potassium chloride solution containing ^{22}NaCl (1 μCi/ml) (final concentration, 1 mM), 1 mM ouabain, and 100 μM bumetanide. In some wells, the uptake solution contained 0.1 mM EIPA. After 1 min, cells were rapidly washed four times with ice-cold phosphate-buffered saline to terminate $^{22}Na^+$ uptake. The pH_i was calculated from the imposed $[K^+]$ gradient by assuming intracellular K^+ concentration of 120 mM.

Measurement of pH_i—Cells were grown on Cellgen (Koken Ltd.) coated plastic coverslips, and a group of cells were serum-depleted for 24 h. Cells were loaded with 1 μM BCECF for 10 min in the buffer (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Tris-Cl, pH 7.4), washed, and immediately mounted on coverslips. As indicated, the medium contained additionally 10% serum and/or 25 mM $NaHCO_3$. BCECF fluorescence was measured at a constant emission wavelength (550 nm) by alternately exciting the dye at 440 and 490 nm on fluorescence spectrophotometer (Spex). pH_i was calibrated in nigericin (25 μM) containing high K^+ buffer (130 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 30 mM Hepes-Cl, adjusted usually to pHs 6.8, 7.0, 7.2, 7.4, and 7.6). As indicated, this calibration solution additionally contained 10% serum and/or 25 mM $NaHCO_3$. Change in pH_i was also measured by the [^{14}C]benzoic acid-equilibration method (Fig. 8*B*) (30). For this measurement, serum-depleted cells were preincubated for 30 min in bicarbonate-free Hepes-buffered Dulbecco's modified Eagle's medium, pH 7.0, and then incubated in the same medium containing [^{14}C]benzoic acid (1 μCi/ml) for 10 min at 37 °C. After washing four times with ice-cold phosphate-buffered saline, ^{14}C radioactivity taken up by cells was measured. Change in pH_i was calculated as described previously (30).

Oocyte Experiment—Xenopus oocytes were stripped and defolliculated enzymatically with 1 mg/ml collagenase in Ca^{2+}-free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM Hepes-NaOH, pH 7.5) for 30 min at room temperature. Defolliculated oocytes were injected with 50 nl of cRNA (50 ng) or DEPC-treated H_2O using a 10-μl micropipette (Drummond Scientific Co.). Injected oocytes were kept for 3 days at 18 °C in 0.5× L-15 solution (1:1 dilution of Leibovits L-15 medium (Invitrogen) in filter-sterilized 50 mM Hepes-NaOH, pH 7.5) containing 50 units/ml nystatin (Invitrogen) and 0.1 mg/ml gentamicin (Invitrogen). Oocytes were preincubated for 1 h in NH_4Cl medium (80 mM NH_4Cl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes-Tris, pH 7.4), washed twice with choline-chloride medium (80 mM choline-chloride, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes-Tris, pH 7.4), and then incubated for 15 min in the same medium additionally containing 1 mM ^{22}NaCl (10 μCi/ml), 1 mM ouabain, and 0 or 0.1 mM EIPA. Oocytes were washed six times with ice-cold non-radioactive choline-chloride medium, and then $^{22}Na^+$ radioactivity was measured.

RESULTS

Human CHP2 protein has a primary sequence highly homologous to those of human CHP1 (NCBI protein accession number Q99653, 61% identity), mouse CHP1 (NCBI protein accession number Q62877, 60% identity), and mouse CHP2 (NCBI protein accession number Q9D869, 80% identity) (Fig. 1*A*). Similar to CHP1, CHP2 contains an N-terminal myristoylation site (Gly-2) as well as four EF-hand Ca^{2+} binding motifs of which two ancestral sites may not bind Ca^{2+} because of the

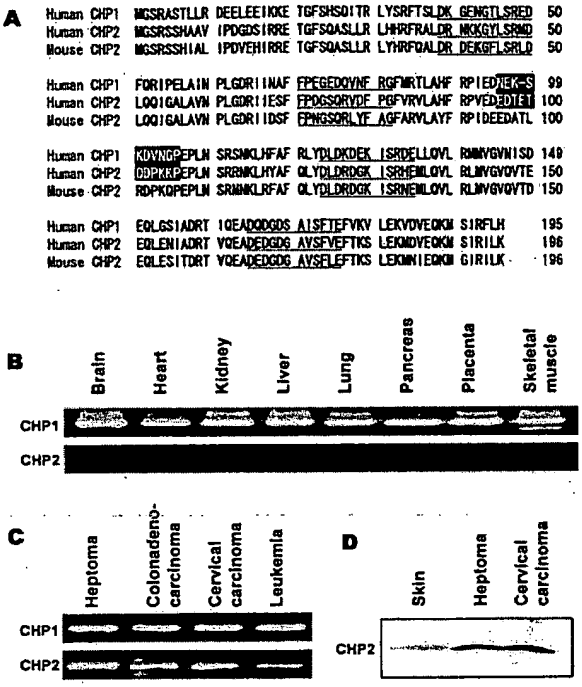

FIG. 1. **Sequence alignment and expression pattern of CHP isoforms.** *A*, amino acid sequences of human CHPs 1 and 2 and mouse CHP2 were aligned. Identical residues were *highlighted*. Four EF-hand Ca^{2+} binding motifs were *underlined* of which N-terminal two ancestral sites do not have a typical EF-hand sequence and thus may not bind Ca^{2+}. Synthetic peptide sequences used for the production of antibodies were marked by a *black box*. *B* and *C*, expression patterns of CHP1 and CHP2 were analyzed by RT-PCR. PCR reactions were performed using sets of primers 5′-tctcgggcctccacgttactgcgggacg-3′ and 5′-ATACTAGACCGCAAGAA CAG-3′ for CHP1 and 5′-CCACGCCTCTCCGGCGGGAGG-3′ and 5′-ATGGGGGCTTTGGA TGAATTC-3′ for CHP2 on templates of cDNA (1 ng) from normal tissues (human MTC panel I) or from malignantly transformed cells as indicated. After 35 cycles of PCR amplification, PCR products were analyzed on a 1% agarose gel. *D*, proteins (50 μg each) prepared from human skin fibroblasts and hepatoma and cervical carcinoma cells were subjected to immunoblot analysis with anti-CHP2 antibody.

substitution of critical acidic residues (Fig. 1*A*). We compared the expressions of CHP1 and CHP2 by RT-PCR using a cDNA panel from normal human tissues, *i.e.* brain, heart, kidney, liver, lung, pancreas, placenta, and skeletal muscle (human MTC™ panel I). PCR bands for CHP1 were detected in all of the human tissues tested (Fig. 1*B*). However, PCR bands for CHP2 were not detected in these tissues even after 35 cycles of PCR amplification (Fig. 1*B*). Consistent with these observations, we failed to detect CHP2 message in normal human tissues including brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, and peripheral blood leukocyte by Northern blot analysis in which commercially available poly(A^+) RNA Northern blot (human 12-line MTN™ blot, Clontech) were hybridized with the ^{32}P-labeled full-length CHP2 probe under high stringency conditions (data not shown). In contrast to normal tissues, on the other hand, RT-PCR readily detected CHP2 as well as CHP1 in several malignantly transformed cells (Fig. 1*C*). The CHP2 expression in some cancer cells was also confirmed by immunoblot analysis (Fig. 1*D*). Taken together, these data suggest that CHP2 is expressed in malignantly transformed cells although rarely expressed in normal tissues or cells.

We previously showed that CHP1 binds to specific juxtamembrane regions (amino acids 510–530 in case of NHE1) of

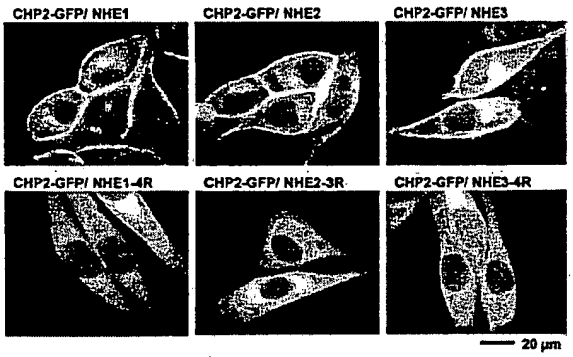

FIG. 2. **Subcellular localization of GFP-tagged CHP2.** CHP2-GFP was expressed in PS120 cells stably expressing indicated NHE variants. Enriched cell populations expressing CHP2-GFP were placed in serum-free Dulbecco's modified Eagle's medium without phenol red. Images were taken under a fluorescent microscope equipped with a CoolSNAP imaging system (RS Photometrics). In NHE1–4R, NHE2–3R, or NHE3–4R, three or four hydrophobic residues Phe^{526}, Leu^{527}, Leu^{530}, and Leu^{531} of human NHE1; Phe^{507}, Phe^{508}, and Val^{511} of rat NHE2; or Ala^{480}, Phe^{481}, Ile^{484}, and Leu^{485} of rat NHE3 were replaced by arginine residues. These mutations were reported to disrupt interaction with CHP1 (6).

the cytoplasmic domains of NHEs 1–4 (6). CHP1 is also likely to bind to a corresponding region in NHE5 because of the high sequence homology of the relevant regions. We examined whether CHP2 binds to these CHP1-binding regions in NHEs 1–3 by observing subcellular localization of GFP-tagged CHP. In the exchanger-deficient PS120 cells, GFP-tagged CHP2 was uniformly distributed in the cytosol (data not shown). In contrast, in cells expressing NHE 1, 2, or 3, a part of CHP2-GFP was localized in the plasma membrane (Fig. 2). However, plasma membrane localization of CHP2-GFP was not detected in cells expressing mutant exchangers NHE1–4R, NHE2–3R, or NHE3–4R, which do not bind CHP1 (Fig. 2) (6), indicating that CHP2 also binds to CHP1-binding regions in NHEs 1–3.

Interaction of CHP2 with NHE1 was also confirmed by pull-down assay using a MBP fusion protein containing the cytoplasmic domain (aa503–815) of NHE1 (Fig. 3*A*). As a negative control, we showed that CHP2 does not bind to a MBP fusion protein containing the cytoplasmic domain (aa500–669) of NHE6 (Fig. 3*B*). These data suggest that CHP2 directly interacts with the juxtamembrane CHP1-binding site in the plasma membrane-type exchangers. To examine the relative binding efficiency of CHP1 *versus* CHP2, we carried out pull-down assay using MBP-NHE1 fusion proteins in the presence of a constant amount of CHP2 and different amounts of CHP1. As shown in Fig. 3*C*, *lower panel*, the amount of CHP2 protein recovered by pull down decreased with increasing amounts of CHP1 by competitive interaction for the common binding site in NHE1. When 250 μg of CHP1 and 50 μg of CHP2 were present, almost equal amounts of CHP1 and 2 were recovered (Fig. 3*C*), suggesting that CHP2 binds to NHE1 more strongly (~5-fold) than does CHP1.

We used the oocyte expression system to examine the role of CHP2 in the exchange activity. As shown in Fig. 4, the injection of cRNA for NHE1 or NHE3 significantly enhanced exchange activity in oocytes. Co-injection of cRNA for CHP1 or CHP2 together with cRNA for NHE1 or NHE3 further enhanced the exchange activity. The data suggest that CHP1 and CHP2 have the ability to increase exchange activities of NHE1 and NHE3 to a similar extent.

To observe the functional difference between CHP1 and CHP2, we produced stable cell clones overexpressing CHP1 or CHP2. Immunoblot analysis revealed that PS120 cells express

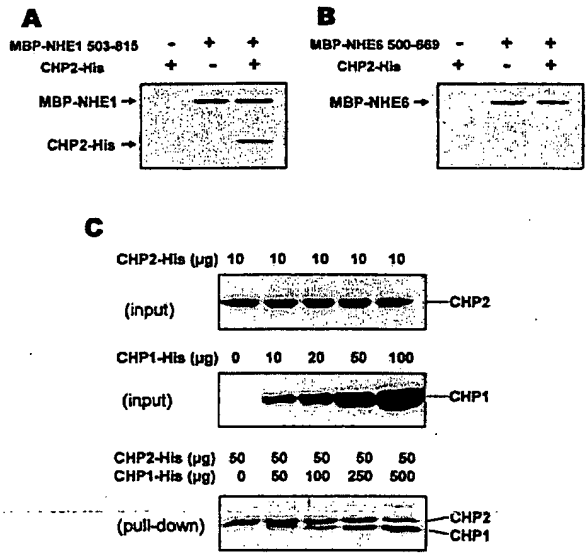

FIG. 3. **Pull-down assay for CHP-NHE interaction.** *A* and *B*, 150 μg of CHP2-His protein was mixed with 30 μl of amylose resin pretreated with 200 μg of MBP-NHE1 or MBP-NHE6 fusion protein, respectively, and incubated for 60 min. After resins were washed, proteins were eluted with 50 mM maltose, electrophoresed, and visualized by Coomassie Brilliant Blue staining. *C*, 50 μg of CHP2-His protein and different amounts of CHP1-His protein were mixed with 30 μl of amylose resin pretreated with 100 μg of MBP-NHE1 fusion protein, incubated for 60 min, eluted, and analyzed by electrophoresis (*bottom panel*). Protein inputs were also shown for reference (*upper* and *middle panels*).

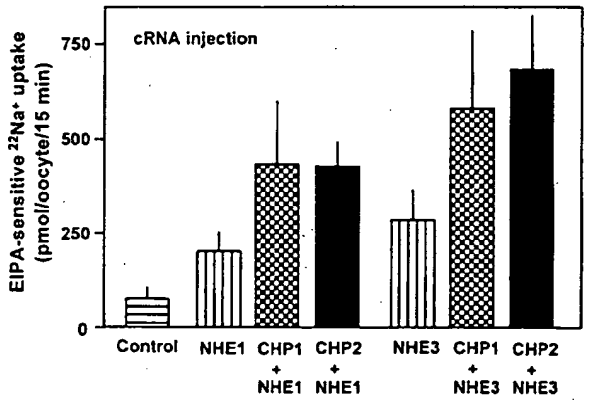

FIG. 4. **Oocyte experiment.** Three days after injection of cRNA into *Xenopus* oocytes, $^{22}Na^+$ uptake was measured in the presence or absence of 0.1 mM EIPA as described under "Experimental Procedures." Data are means ± S.D. of data from 30 oocytes.

a relatively high level of endogenous CHP1 and a very low level of endogenous CHP2 as compared with the respective proteins overexpressed in the same cells (Fig. 5). Anti-CHP1 immunoprecipitated NHE1 protein from cells co-expressing CHP1/NHE1 but not from cells co-expressing CHP2/NHE1, whereas anti-CHP2 immunoprecipitated NHE1 protein from cells co-expressing CHP2/NHE1 but not from cells co-expressing CHP1/NHE1 (Fig. 5). These antibodies immunoprecipitated much lower levels of NHE1 protein from NHE1 transfectants not expressing exogenous CHP1 or CHP2 (Fig. 5), consistent with the above finding that PS120 cells express endogenous CHP1 and CHP2. The NHE1 protein was not detected in the immunoprecipitated material obtained with anti-CHP2 from

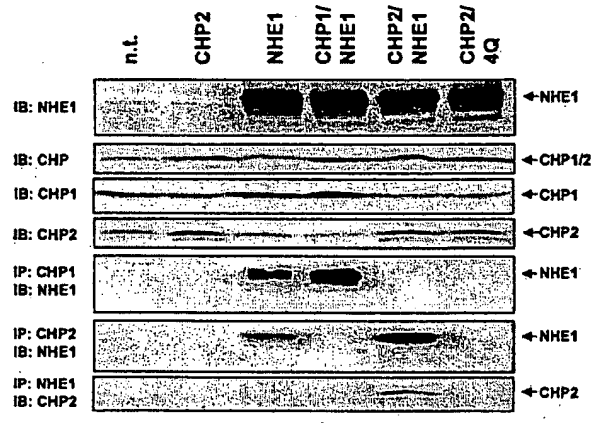

FIG. 5. **Co-immunoprecipitation analysis of CHP/NHE1 interaction.** CHPs and NHE1 variants expressed stably in PS120 cells are shown above the *panels*, whereas antibodies used are shown on the *left side* of each *panel*. In 4Q, four hydrophobic residues, Phe^{526}, Leu^{527}, Leu^{530}, and Leu^{531} of NHE1, were replaced by four glutamine residues. In the experiments for the *top four panels*, 20-μg proteins from total cell homogenate were analyzed by immunoblotting (*IB*). In the experiments for the *last three panels*, cell lysates were subjected to immunoprecipitation (*IP*) with indicated antibodies and then analyzed by immunoblotting as described under "Experimental Procedures." *n.t.*, non-transfected cells.

cells not expressing NHE1 (Fig. 5, *n.t.* or *CHP2*) or cells expressing a CHP-binding-defective NHE1 (Fig. 5, *4Q*). These data suggest that exogenous CHP1 or CHP2 replaces endogenous CHP bound to NHE1 protein.

We examined the effect of 24-h serum depletion on exchange activity in cells co-expressing CHP1/NHE1 or CHP2/NHE1 measured at different pH_i values. Both groups of cells exhibited high $^{22}Na^+$ uptake activity (50–60 nmol/mg/min) at low pH_i (5.6), independent of serum depletion (Fig. 6*A*). In CHP1/NHE1 cells, exchange activity at a neutral pH_i (6.8–7.2) was significantly lower with serum depletion than without serum depletion (Fig. 6, *B–D*). In sharp contrast, prior treatment with or without serum did not affect exchange activity significantly in CHP2/NHE1 cells at all pH_i values tested (Fig. 6, *B–D*). Consistent with $^{22}Na^+$ uptake, the resting pH_i in CHP2/NHE1 cells was significantly elevated (7.4–7.5) in the absence (Fig. 7*A*) or presence (Fig. 7*B*) of bicarbonate regardless of prior treatment with or without serum, whereas pH_i was significantly lower in CHP1/NHE1 cells subjected to serum deprivation. Thus, NHE1 was constitutively activated in cells expressing CHP2/NHE1. Of note, long serum depletion significantly reduces pH_i in CHP2 transfectants not expressing NHE1 (Fig. 7) or in cells co-expressing the CHP-binding-defective NHE1 mutant 4Q and CHP2 (data not shown). Such an effect was also observed in non-transfected PS120 cells (data not shown), suggesting that relatively long serum depletion may affect other pH_i-regulating systems.

We next examined the acute effect of serum addition on pH_i of cells that had been maintained for 24 h under serum depletion. Serum induced a relatively large cytoplasmic alkalinization in CHP1/NHE1 cells as measured by BCECF fluorescence (Fig. 8*A*) or ^{14}C-benzoic acid equilibration method (Fig. 8*B*). Cytoplasmic alkalinization occurred similarly in NHE1 cells or in CHP1/NHE1 cells (Fig. 8*B*). In contrast, serum addition caused a minimum alkalinization in CHP2/NHE1 cells. Alkalinization was not observed in CHP2 transfectants not expressing NHE1 or in CHP2 transfectants expressing 4Q (Fig. 8, *A* and *B*). These results confirmed that NHE1 was already activated in CHP2/NHE1 transfectants maintained in the absence of serum.

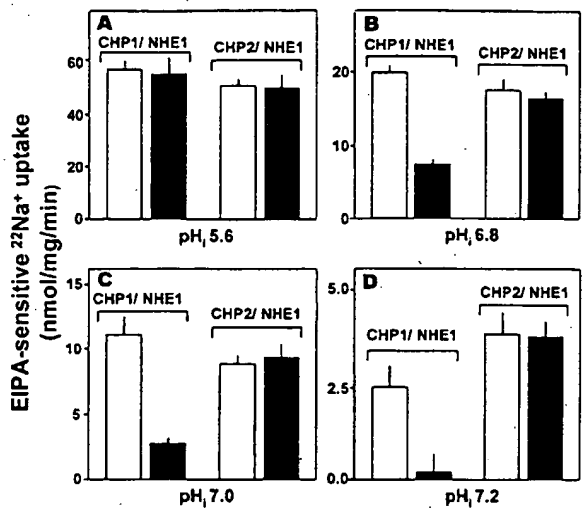

FIG. 6. **The effect of serum depletion on $^{22}Na^+$ uptake activity.** PS120 cells stably co-expressing CHP1/NHE1 or CHP2/NHE1 grown in 24-wells were serum-depleted for 24 h. The pH_i values of serum-supplemented (*open bars*) or serum-depleted cells (*closed bars*) were clamped at 5.6, 6.8, 7.0, or 7.2 by incubating cells in the solutions containing nigericin and the appropriate concentrations of KCl, and then EIPA-sensitive $^{22}Na^+$ uptake was measured as described under "Experimental Procedures." Data are means ± S.D. (n = 9).

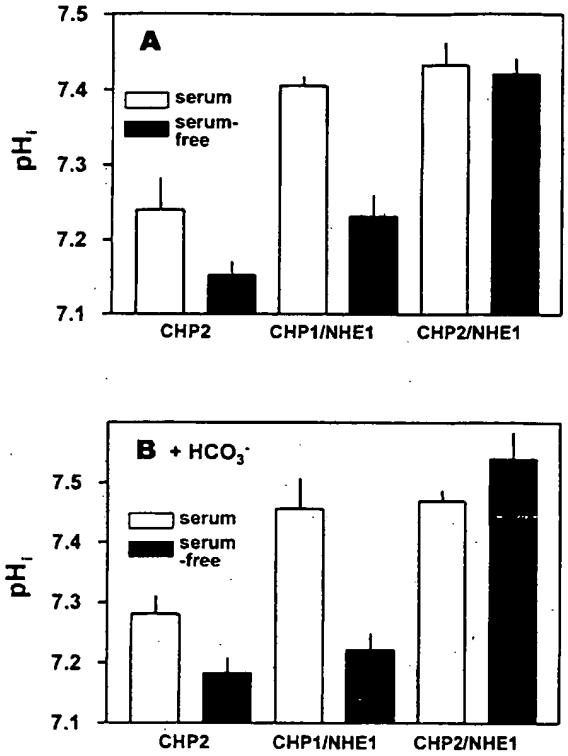

FIG. 7. **The effect of serum depletion on the resting pH_i.** The resting pH_i of PS120 cells expressing CHP2, CHP1/NHE1, or CHP2/NHE1, which were maintained for 24 h in the presence (*open bars*) or absence (*closed bars*) of serum, was measured in the absence (*A*) or presence (*B*) of 25 mM $NaHCO_3$ by monitoring BCECF fluorescence as described under "Experimental Procedures." Data are means ± S.D. (n = 9).

Intracellular pH has been reported to influence both cell growth (4) and cell viability (20–23, 33–36). Indeed, cell number increased efficiently in the presence of serum upon expression of NHE1 regardless of the type of CHP isoform co-expressed, whereas cells expressing 4Q grew relatively slowly (Fig. 9*A*). Despite this apparently similar role of CHP1 and CHP2 in cell growth, the ability to maintain cell viability under serum starvation was dramatically different between cells expressing NHE1/CHP1 and NHE1/CHP2 (Fig. 9*B*). We evaluated cell viability by counting the number of cells remained attached to dishes during serum starvation. These cells excluded trypan blue and were able to restart growth upon re-addition of serum. Cells expressing CHP2/NHE1 were significantly more resistant to serum starvation ($t_{1/2}$ = ~14 days) than cells expressing CHP1/NHE1 ($t_{1/2}$ = ~4 days). Surprisingly, 60% of cells expressing CHP2/NHE1 were still viable 10 days after serum starvation when all CHP1/NHE1 cells lost viability (Fig. 9*B*). The high viability of CHP2/NHE1 cells required enhanced NHE1 activity but not the expression of CHP2 itself, because both CHP2 or CHP2/4Q cells were very sensitive to serum starvation ($t_{1/2}$ = ~2 days) (Fig. 9*B*) and because EIPA markedly accelerated the loss of viability in CHP2/NHE1 cells (Fig. 9*C*). These data suggest that the high viability of CHP2/NHE1 cells may be the result of high pH_i caused by high Na^+/H^+ activity achieved in these serum-starved cells.

DISCUSSION

In this study, we characterized the function of CHP2 that shares a high sequence homology with CHP1. We previously reported that CHP1 serves as an essential cofactor, supporting the physiological activity of plasma membrane-type Na^+/H^+ exchangers (6). CHP2 exerts similar effects. (i) GFP-tagged CHP2 co-localized with NHEs 1–3 but not with CHP1-binding-defective mutants NHE1–4R, NHE2–3R, or NHE3–4R in the plasma membrane. (ii) Recombinant CHP2 was bound to a MBP fusion protein containing the NHE1 cytoplasmic domain but not the fusion protein containing NHE6 cytoplasmic domain. (iii) CHP2 enhanced exchange activities of NHE1 and NHE3 when co-expressed with them in oocytes. (iv) The V_{max} of CHP2/NHE1 was comparable with that of CHP1/NHE1, whereas CHP2/4Q cells exhibited a very low exchange activity (<10%) compared with CHP2/NHE1.

Intriguingly, CHP1/NHE1 and CHP2/NHE1 cells responded differently to serum depletion, although they exhibited a similar high exchange activity when maintained in serum. In the absence of serum, CHP2/NHE1 exhibited a higher steady-state pH_i and higher exchange activity in the neutral pH_i range as compared with CHP1/NHE1. Thus, NHE1 becomes activated with bound CHP2 independent of serum. We observed that steady-state levels of pH_i in CHP1/NHE1 and CHP2/NHE1 cells maintained for 24 h in serum-supplemented or serum-depleted medium were not affected by the presence or absence of bicarbonate. In contrast, the presence of bicarbonate influenced the response of CHP1/NHE1 cells to a short incubation (10–20 min) with serum. The pH_i of CHP1/NHE1 was elevated in response to acute exposure to serum in the absence of bicarbonate (Fig. 8), but this did not happen in the presence of bicarbonate (data not shown). Such an inhibitory effect of bicarbonate on pH_i may be because of activation of the anion exchanger. At present, however, it is not clear why bicarbonate did not exhibit an inhibitory influence on pH_i in CHP1/NHE1 and CHP2/NHE1 cells chronically exposed to serum (Fig. 7).

We found that CHP2/NHE1 cells are highly viable even under a long term serum starvation. Ubiquitous CHP1 was previously suggested to be involved in various cell functions, such as inhibition of calcineurin activity (37), vesicular transport of proteins (38), interaction with microtubules (39), and interaction with a death-associated protein kinase-related

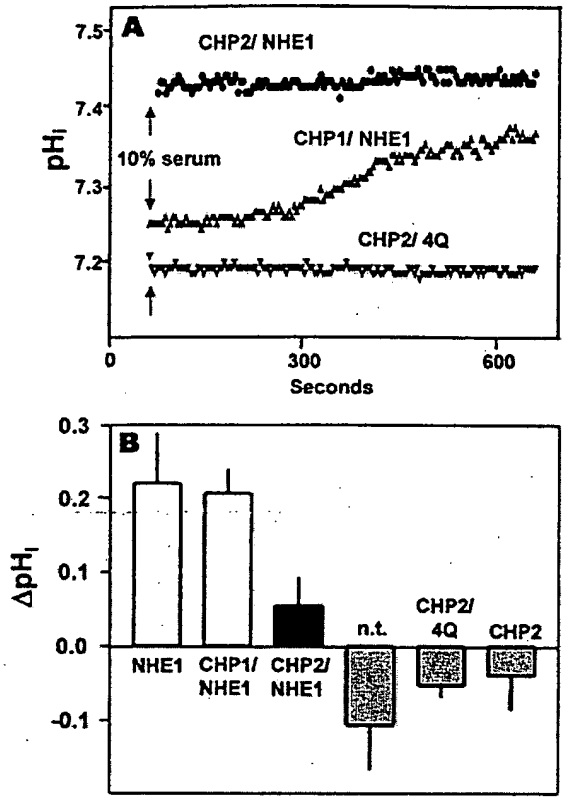

FIG. 8. **Serum-induced acute change in pH$_i$ of cells expressing CHP1 or CHP2.** *A*, serum-depleted cells were loaded with 1 μM BCECF-AM, washed, and set in fluorescent spectrophotometer. At the time indicated by an *arrow*, 10% dialyzed serum was added. Change in BCECF fluorescence was measured as described under "Experimental Procedures." *B*, change in pH$_i$ was measured by ^{14}C-benzoic acid equilibration method as described under "Experimental Procedures." Change in pH$_i$ was measured 10 min after the addition of 10% dialyzed serum. Data are means ± S.D. (n = 3). *n.t.*, non-transfected.

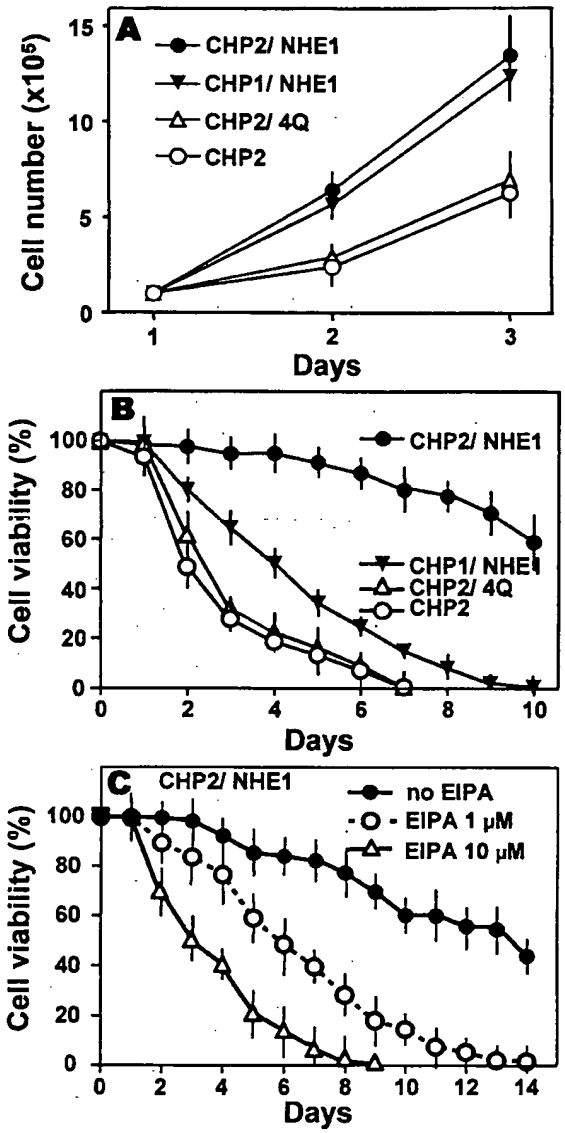

FIG. 9. **Effect of expression of CHP1 or CHP2 on cell growth and cell viability under serum starvation.** *A*, growth rate of various transfected cells was measured in the presence of 7.5% serum. At the indicated time, cells were trypsinized and counted by hemocytometer. *B* and *C*, cell viability under serum starvation. The same numbers of cells were seeded on 60-mm dishes, and 1 day later, serum was removed. *C*, in some dishes, the medium contained 1 or 10 μM EIPA. We counted the number of cells that remained attached in four 5 × 5-mm square areas/each dish. We counted the cell numbers in the same areas throughout experiments and plotted them in the figure. Three dishes were used for respective transfectants. Data are means ± S.D. (n = 12).

apoptosis-inducing protein kinase 2 (DRAK2) (40). Although we do not know whether CHP2 is also involved in these cellular functions, they may not be relevant to the observed effects of CHP2 because the functions of CHP1/NHE1 and CHP2/NHE1 were compared in this study. However, we cannot rule out a possibility that cellular functions specific to CHP2 may be involved in the observed CHP2 effect. At any rate, it is probable that high viability of CHP2/NHE1 cells results from maintenance of high pH$_i$ through serum-independent activation of NHE1, because cells overexpressing CHP2 were sensitive to serum starvation when active NHE1 was not expressed or when EIPA was present in the medium. Factors such as ultraviolet light, Fas ligand, somatostatin, and IgM are known to induce cell acidification, which precedes apoptotic cell death in various cell types such as Jurkat cells (33), HL-60 leukemia cells (34, 36), human B lymphomas (21, 22), and human breast cancer cells (20, 35). Our data also suggest that pH$_i$ is an important determinant for serum deprivation-induced cell death.

We found that relatively high levels of CHP2 mRNA were detected in several types of malignantly transformed cells but not in normal tissues or cells. This is consistent with previous findings that CHP2 expression was detected in liver carcinoma cells (GenBank™ nucleotide accession number AF146019) and colon tumor metastatic cells (GenBank™ nucleotide accession number EST370271). It has been well documented that malignantly transformed cells maintain abnormally high pH$_i$. For example, microinjection of *ras* p21 protein (25) and stable transfection of Ha-*ras* oncogene (26, 27) or E7 oncogene of papillomavirus type 16 (28) resulted in a marked increase in the steady-state pH$_i$ in NIH-3T3 cells through the activation of NHE1. Moreover, high pH$_i$ because of activation of NHE1 has been observed in various malignantly transformed cells, such as human leukemic (21), human malignant glioma (41), and human breast cancer cells (42). Interestingly, in this latter study (42), pH$_i$ was shown to be higher under serum-deprived

rather than under serum-supplemented conditions. All of these studies suggest that NHE1 becomes permanently activated in many malignantly transformed cells, although the molecular mechanism for such a phenomenon remains unclear. The properties of NHE1 in these transformed cells are similar to those of CHP2/NHE1 observed here. CHP2 appears to be almost exclusively expressed in these transformed cells. In addition, NHE1 interacts more strongly with CHP2 than with CHP1. Therefore, we propose that the activation of NHE1 by bound CHP2 may be a key mechanism for the maintenance of serum-independent high pH_i in these abnormal cells.

At present, we do not know how NHE1 is activated by CHP2. A previous study (5) suggested that serum changes the phosphorylation state of CHP1. Although it is not clear whether phosphorylation of CHP1 is a key event in the growth factor-induced activation of NHE1 in normal cells, the difference in the phosphorylation status could be one possible mechanism to explain the observed difference in the mode of serum-dependent regulation of NHE1 by CHP1 or CHP2. Indeed, there are several potential phosphorylation sites that differ between CHP1 and CHP2. For example, potential phosphorylation sites for protein kinase C (Ser^{112}) or calmodulin-dependent protein kinase II (Thr^{7}, Ser^{33}, and Ser^{37}) in CHP1 are not conserved in CHP2. Alternatively, these CHP isoforms may interact with different proteins that mediate different signals from serum to NHE1·CHP complex. Further studies including analyses with chimeric or mutated CHPs and determination of the crystal structure of NHE1·CHP complex are required to elucidate the molecular mechanism in the generation of the functional difference between CHP1 and CHP2.

In summary, the present data suggest that the interaction of NHE1 with CHP2 leads to serum-independent permanent activation of NHE1, which in turn results in the protection of cells from serum deprivation-induced death. A recent study (28) has reported that NHE1 inhibitor markedly retarded the development of tumor in nude mice. Our study suggests that CHP2 may be a novel important target for anticancer therapy as it appears to be almost exclusively expressed in malignantly transformed cells.

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